The effect of the treatment of denture related stomatitis on peripheral T cells and monocytes.

Joanna Maciąg[1], Tomasz Mikołajczyk[2], Paweł Matusik[2], Daniel Nowakowski[1], Douglas Robertson[4], Anna Maciąg[3], Grzegorz Osmenda[2], Marta Cześnikiewicz-Guzik[1,4]

[1]Institute of Dentistry, Department of Dental Prophylaxis and Experimental Dentistry, Jagiellonian University Medical College, Cracow, Poland;
[2]Department of Internal and Agricultural Medicine, Jagiellonian University Medical College, Cracow, Poland;
[3]Zbigniew Żak Voivodeship Dental Clinic, Cracow, Poland;
[4]Oral Sciences Research Group, Glasgow Dental School, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow

Author contribution and address:

Joanna Maciąg, MSc	Blood preparation, flow cytometry tests, analysis of flow cytometry results, statistical analysis, manuscript writing	Institute of Dentistry, Department of Dental Prophylaxis and Experimental Dentistry, Jagiellonian University Medical College, ul. Montelupich 4, 31-155 Cracow, Poland
Tomasz Mikołajczyk, PhD	Analysis of flow cytometry results	Department of Internal and Agricultural Medicine, Jagiellonian University Medical College, ul. Skarbowa 1, 31-121Cracow, Poland
Paweł Matusik, MD, PhD	Flow cytometry tests (intracellular staining), analysis of flow cytometry results	Department of Internal and Agricultural Medicine, Jagiellonian University Medical College, ul. Skarbowa 1, 31-121Cracow, Poland
Daniel Nowakowski, DMD, DDS	Patients recruitment	Institute of Dentistry, Department of Dental Prophylaxis and Experimental Dentistry, Jagiellonian University Medical College, ul. Montelupich 4, 31-155 Cracow, Poland
Douglas Robertson, PhD BDS (Hons) MFDS RCSEd FHEA	Manuscript proofreading	Infection and Immunity Research Group, Glasgow Dental School, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, 378 Sauchiehall Street, Glasgow, UK
Anna Maciąg, DMD, MD	Patients recruitment	Zbigniew Żak Voivodeship Dental Clinic, ul. Batorego 3, 31-135 Cracow, Poland
Grzegorz Osmenda, MD, PhD	Patients demographic information collection	Department of Internal and Agricultural Medicine, Jagiellonian University Medical College, ul. Skarbowa 1, 31-121Cracow, Poland
Marta Cześnikiewicz-Guzik, DMD, PhD	Idea, experimental design, patients recruitment, manuscript writing and proofreading	1. Institute of Dentistry, Department of Dental Prophylaxis and Experimental Dentistry, Jagiellonian University Medical College, ul. Montelupich 4, 31-155 Cracow, Poland 2. Infection and Immunity Research Group, Glasgow Dental School, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, 378 Sauchiehall Street, room F15, Glasgow, UK

Corresponding author:
Marta Czesnikiewicz-Guzik
[4]Infection and Immunity Research Group, Glasgow Dental School, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow
378 Sauchiehall Street, room F15
Email: marta.czesnikiewicz-guzik@glasgow.ac.uk
Phone: 004407557674687

Abstract

Purpose: Systemic immune activation has been recently linked to chronic inflammatory disorders of the oral cavity, particularly periodontitis. In our study we aimed to determine whether treatment of fungus induced oral inflammation, namely denture-related stomatitis (DRS) can affect the activation of the systemic immune response.

Materials and Methods: Peripheral blood from patients with denture-related stomatitis caused by *Candida albicans* infection (n = 15) was collected at three time points: before treatment with nystatin, at the end of therapy and 2 months after finishing therapy. Activation of T cells and monocytes was assessed by flow cytometry.

Results: We found that the percentages of peripheral lymphocytes, T cells and their subpopulations, as well as monocytes were similar before, immediately following and two months after nystatin treatment. Cells expressing early activation marker CD69 and RANTES C-C chemokine receptor type 5 were significantly increased immediately after treatment and returned to baseline levels after two months. Th17 cells, which were implicated in the pathogenesis of DRS remained unchanged. Central memory CD4+ subset and intermediate subset of monocytes were lower after therapy and this effect was sustained for two months.

Conclusion: Treatment of denture-related stomatitis does not seem to affect the general state of the cellular components of the immune system. Our results suggest a potential proinflammatory effect of the antifungal agent, nystatin. Although transient and non intense, this effect might be of particular clinical importance, because of relationships between inflammation and certain diseases. Further studies are required to clarify this aspect.

Keywords: Candida albicans; denture-related stomatitis; nystatin; lymphocytes; monocytes

CCR5 - C-C chemokine receptor type 5

DRS - denture-related stomatitis

IL - interleukin

PBMC – peripheral blood mononuclear cells

SD - standard deviation

Th - T helper cells

Q1 and Q3 - 25th (Q1) and 75th (Q3) percentiles

Introduction

Denture-related stomatitis (DRS) is a condition where inflammation of the oral mucous membrane occurs beneath a denture. The disease is often known as Candida-associated denture induced stomatitis as opportunistic infection with Candida species is associated in about 90% of cases [56]. DRS is one of the most common diseases in the elderly, affecting up to 70% of patients in the course of life, making it an important clinical issue. DRS can negatively influence the patients' quality of life, especially in edentulous subjects wearing complete dentures [1,12,23,44]. Clinical signs of DRS include erythema and swelling of palatal mucosa, sometimes combined with subjective symptoms, such as dysgeusia or burning sensation. The aetiology of the DRS is multifactorial [12]. Long-term and continuous use of dentures and poor denture and oral hygiene habits promote the development of a biofilm, called denture plaque, on the surface of the prosthesis [12,44]. *Candida albicans* is component of the normal microflora of the human oral cavity [3,13], however, in the presence of a denture and accompanied by favourable conditions, including low salivary pH, regular sugar consumption [29] and local immune surveillance changes, like decreased salivary antimicrobial enzymes activities and increased transforming growth factor β and nitric oxide levels [11], *C. albicans* becomes an opportunistic pathogen leading to infection and DRS.

It was recently reported that DRS was related to selected systemic co-morbidities, such as vascular dysfunction [26]. However, the mechanisms linking oral infection and vascular defects are not yet known. While in periodontitis systemic activation of the immune system [15,47] is established to be very important in mediating increased cardiovascular risk, the extent of systemic activation of immune response to DRS is poorly characterized.

It is known, that the presence of oral fungal infection influences the local immune mechanisms in oral cavity in a variety of ways. The mucosal immune response to fungal pathogens include neutrophils as major effectors of the innate response, driven by cytokines released from epithelial cells after detection of pathogen [46]. Epithelial cells also produce β-defensin 2 and chemokine (C-C motif) ligand 20, which orchestrate dendritic cell recruitment [34]. Primed dendritic cells travel to the local lymph nodes and present fungal antigens to the T cell, which can differentiate to the T helper (Th): Th1, Th2, Th17 and T regulatory (reg) cells. Th17 and Treg cells play a vital role in fighting fungal infection [10,34]. However, it still remains unclear if these local immune responses are reflected by changes in systemic immunity. There are limited studies suggesting that oral *Candida* infection may impact on the peripheral blood mononuclear cells (PBMC) state, as measured by levels of cytokines produced *in vitro* in response to *Candida* antigens [36,43]. The existence of changes in peripheral blood populations that are known to be related to systemic chronic inflammation such as CD16+ monocytes [22,49] or activated T cells is not known.

The relationship between oral *Candida albicans* infection and the systemic inflammatory response has not yet been studied. The aim of this study therefore was to determine, whether the treatment of local inflammation caused by denture-related stomatitis influenced the systemic immune response as measured in activation of peripheral blood immune cells.

Methods

The study was independently reviewed and approved by local ethics committee of Jagiellonian University. Written informed consent was obtained from all patients and study was conducted in accordance with the Declaration of Helsinki. This study has been registered in ClinicalTrials.gov (identification no. NCT02166450).

Patients and clinical evaluation: Patients (n = 15) using acrylic dental prosthesis for at least 6 months presenting with clinical signs of DRS such as: erythema, swelling, discomfort and pain in the palatal area were recruited. DRS was graded clinically according to Newton's classification scale. Swabs from the inflamed area have been taken for the routine microbiological laboratory diagnostic tests for *Candida* species presence to confirm the clinical diagnosis. Swabs were taken from the hard palate (between the second and third palatal fold), after an overnight fast and after at least 6 hours of denture wear, without cleaning, use of adhesives or rinsing the mouth with disinfectants. Patients were recruited when clinical symptoms were confirmed with microbiological culture. In patients with teeth present, a periodontal examination was carried out to exclude patients with periodontitis (patients excluded if periodontal pocket depths ≥4 mm were identified). Exclusion criteria included also acute inflammatory disorders other than DRS, neoplastic disease relapses or chemotherapy courses less than 5 years before the enrolment, using antibiotics in the previous 4 weeks or anti-inflammatory drugs (steroids and non-steroidal, excluding aspirin in doses less than 80 mg) in the previous 2 months prior to enrolment. Patients with a history of myocardial infarction, acute coronary incidents, vascular inflammation, chronic haematological disorders and immunodeficiency or major medication changes in the 5 weeks prior to enrolment, or during the study were also excluded. Patients were recruited consecutively and all subjects, who agreed to participate in the study and completed three study visits were included in the analysis.

Patients were referred for treatment of fungal infections, which included application of nystatin (100 000 IU) every 6 h for 2 weeks on the mucosal surface of the dentures. Denture that had been used for more than 5 years were replaced. In this case the patient was applying nystatin during first week on the old denture and during second week on the new denture. If the denture was new and did not require replacement, patients were advised to soak the denture in a solution of Corega™ tablets as the daily care, in accordance with the manufacturer's instructions. Blood samples were collected from all patients during three visits: the first sample was collected before introducing treatment regimen (visit 1), the second immediately after treatment to assess direct influence of therapy (visit 2), and the third sample was taken 2 months after completion of treatment to assess longer-term effects of termination of infection (visit 3).

Information about systemic risk factors of DRS such as diabetes (fasting glucose level ≥7 mmol/L or HbA1c >6.5% or current treatment with insulin or oral hypoglycemic agents), and smoking (current or within last 6 months) were based on patient medical records and detailed patient history collected during first visit. Definition of clinical risk factors was based on Perk et. al. [38]

Cytometric analysis: Blood samples were collected in EDTA containing tubes (Becton Dickinson (BD), Franklin Lakes, NJ, USA). PBMC were isolated using Lymphocyte Separation Medium LSM 1077 (PAA Laboratories GmbH, Pasching, Austria). After isolation cells were washed twice with phosphate-buffered saline with heat inactivated 1% fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA) and then incubated for 20 minutes at 4°C with fluorescently labelled monoclonal antibodies (BD

Pharmingen, San Diego, CA, USA). After washing, cells were re-suspended in phosphate-buffered saline with 1% fetal bovine serum and studied on a FACS Canto II or FACS Verse cytometer (BD Bioscences, San Jose, CA, USA). Results were analyzed using FlowJo vX software (FLOWJO LCC Data Analysis Software, Ashland, OR, USA).

Lymphocytes were identified among PBMC on FSC/SSC scatter plots and T cell presence was confirmed by CD3 staining. Percentages of naive (CD45RA+) and memory (CD45RO+) and CD4+ and CD8+ T cells were assessed, as well as presence of surface activation markers in these subpopulations. Monocytes were also identified on FSC/SSC scatter plots with confirmation of CD14/HLA-DR staining. Three main populations were assessed among monocytes: CD14highCD16-, CD14dimCD16+ and CD14highCD16+. The following monoclonal antibodies were used: anti-CD3-PerCP (clone SK7), anti-CD45RA/CD45RO/CD3/CD4 BD Multitest™, anti-CD4-APC (clone RPA-T4), anti-CD4-PeCy7 (clone SK3), anti-CD8-APCH7 (clone SK1), anti-CCR7-PECy7 (clone 3D12), anti-CCR5-PE (2D7/CCR5), anti-CD69-FITC (clone FN50), anti-CD25-PE (clone MA-251), anti-CD28-APC (clone CD28.2), anti-CD14-APCH7 (clone MφP9), anti-HLA-DR-PeCy7 (clone L243), anti-CD16-PE (clone 3G8). Because of technical reasons, it was impossible to perform monocyte analysis in one DRS patient.

Intracellular staining: Intracellular staining was performed in the subgroup of 10 DRS patients. Cytokine production by T cells was assessed after 4-hour incubation of PBMC in RPMI medium 1640 (Gibco, Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA), gentamycin and L-glutamine (Sigma, Saint Louis, MO, USA) with stimulation with Leukocyte Activation Cocktail with BD GolgiPlug (BD Pharmingen, San Diego, CA, USA). After 20 minutes on ice staining for cell surface markers (CD3, CD4 and CD8; procedure, suppliers and clones presented above), cells were washed with phosphate-buffered saline with heat inactivated 1% fetal bovine serum. Cells were permeabilized (20 minutes, on ice) (BD Cytofix/Cytoperm, Fixation and Permeabilization Solution, BD Pharmingen, San Diego, CA, USA) and, after washing with BD PermWash Buffer (BD Pharmingen, San Diego, CA, USA), stained for intracellular cytokines for 20 minutes on ice. Then the cells were washed again with BD PermWash Buffer, resuspended in phosphate-buffered saline with 1% fetal bovine serum and studied, as described for extracellular staining procedure.

The following monoclonal antibodies were used in intracellular staining procedure: anti-IL-17-PE (clone N49-653), anti-IL-4-PE (clone 8D4-8), anti-TNFα-FITC (clone MAb11) and anti-IFNγ-FITC (clone B27). Non-specific staining with isotype-matched control monoclonal antibodies was used (PE Mouse IgG1κ Isotype Control, clone MOPC-21 and FITC Mouse IgG1κ Isotype Control, clone MOPC-21). All antibodies were from BD Pharmingen, San Diego, CA, USA.

Statistical analysis: Assessment of normality of distribution for all continuous variables was conducted using the Shapiro-Wilk test. Variables whose distributions were not consistent with a normal distribution are presented as median and 25th (Q1); 75th (Q3) percentiles and values for subsequent visits were compared by Friedman test, with post-hoc analysis in case of detecting significance. Variables with normal distribution are presented as mean ± standard deviation (SD). All variables with normal distributions described baseline group characteristic and comparison between visits was not applicable. Method of presentation of results is given for each variable in the text. Null hypothesis was "Treatment of DRS does not influence the percentages of total lymphocytes and T cells in peripheral blood of patients with DRS". Values of $p < 0,05$ were considered statistically significant. All analysis were performed with Statsoft Statistica software (StatSoft, Inc., Tulsa, OK, USA).

Results

Study population characteristics: The clinical characteristics of study participants are shown in Table 1. The subjects were mostly women (n = 14; 93.33%) and elderly (mean±SD age 62.5±6.3). The mean BMI was 29.2 (SD 6.1) and 33.3% of patients were current smokers and 33.3% suffered from diabetes mellitus. 93.3% of patients were hypertensive, out of which in only 43% hypertension was controlled. Most of the screened subjects were edentulous, only 4 subjects were partial dentures users who had periodontal examination preformed to exclude any periodontal diseases. Compared to the general population, the proportion of females was significantly higher, what is of particular significance, as these features are key risk factors for DRS.

Lymphocyte characterization in peripheral blood of DRS patients after treatment: The percentages of total lymphocytes and T cells in PBMC of DRS patients showed no significant differences after treatment (Figure 1 upper panel). Similarly no differences were observed in CD4+ and CD8+ T cell subsets (Figure 1 lower panel). Treatment of DRS did not seem to affect median percentages of naive (CD45RA+; 46.8±14.6, 42.5±13.2 and 44.8±13.3; $p = 0.94$) and memory (CD45RO+; 41.13±14.6, 48.72±13.6 and 47.5±13.12; $p = 0.63$) T cells, however a tendency to decrease the percentage of naive and increase the percentage of memory T cells immediately after treatment was observed. Consistently, the percentage of central memory (CD45RA-CCR7+) CD4+ T cells was observed to decrease after treatment, reaching statistical significance for comparison of first and third visit (Figure 2). A similar, albeit non significant, trend was observed for CD8+ memory T cell subset. Percentages of the naive (CD45RA+CCR7+) and effector memory (CD45RA-CCR7-) T cells from both, CD4+ and CD8+ populations, did not show any differences during all three visits (Figure 2).

Lymphocyte activation in peripheral blood of DRS patients after treatment: Interestingly, we found that the median percentages of cells with expression of early activation marker - CD69 in CD3+ cells were significantly higher immediately after treatment and returned to baseline level after 2 months, reaching statistical significance for comparison of first and second and first and third visit, and in CD8+ subset with differences between first and second visit (Figure 3A). In the CD4+ CD69+ subset as well as in subpopulation of cells expressing late activation marker CD25 and with high expression of this marker such pattern was not observed (Figure 3 A, B). What is more, the median percentages of T cells expressing C-C chemokine receptor type 5 (CCR5), the receptor for the chemokine RANTES, in DRS patients were also significantly different between subsequent visits. This percentage increased immediately after treatment and returned to baseline level after 2 months, reaching statistical significance for comparison of 2nd and 3rd visit (Figure 4). The same trend, with significant difference between last two visits, was observed for CCR5-expressing CD4+ and CD8+ subsets, in contrast with percentages of CD4+ and CD8+ CD28null subsets, which were similar during consequent visits (Figure 4).

T cell cytokine production after treatment of DRS: To investigate whether DRS treatment influence major proinflammatory cytokine production in peripheral blood T cells, we performed intracellular staining in the subgroup of 10 patients. We did not observe changes of percentage of T cell subsets producing intracellular IL-17, IL-4, tumour necrosis factors alpha or interferon gamma during subsequent visits (Figure 5).

Monocyte characteristics in the peripheral blood of DRS patients after treatment: We found that there were no statistically significant differences in the percentage of monocytes present in the peripheral blood of patients during subsequent visits (Figure 6, upper panel). As peripheral blood monocyte subpopulation changes might also reflect systemic immune status in chronic inflammation, we next analysed monocyte subsets based on CD14+ and CD16+ expression. We observed, that the percentage of classical CD14highCD16- subset of monocytes decreased immediately after treatment and lower level sustained two months after treatment. The percentage of intermediate CD14highCD16+ monocytes significantly increase after treatment, while non-classical CD14dimCD16+ subset do not show significant changes during subsequent visits. (Figure 6 lower panel).

Discussion

DRS is one of the most common oral diseases among elderly denture wearers [12,23], especially among women [12], smokers and diabetic patients [6]. These epidemiological facts are reflected by population structure in the presented study. The vast majority of recruited patients were female, while smokers and diabetics consisted of as much as one third of study population each. These numbers are high compared to the general population, since 13.6% of diabetics among older adults were reported for Germany [7] or 4.9% for Switzerland [18] and 8.6% of smokers among elderly women for Europe [25].

In our study we aimed to describe the changes in activation of immune system mechanisms evoked by treatment of *Candida* infection in denture-related stomatitis. Since DRS is a clinical issue of increasing importance due to the ageing of population, detection of any association with activation of not only local, but also systemic immunity, is of growing relevance.

Since our experiments are performed in relatively small number of patients, our results should be considered preliminary. A potential weakness of our study is lack of a control group of the patients who did not undergo DRS treatment. This was caused by the fact that the local ethics committee considered it inappropriate to leave patients untreated for the duration of the study. However, we believe that it is very unlikely that we would have observed any changes in untreated patients as the infection is long lasting and well established. This paper represents a significant contribution to the current understanding of the systemic, cellular immune response to the presence of oral inflammation related to the DRS as there is currently very little published data in this area. One should also bear in mind that over 90% of our study group were women, which, although consistent with epidemiology of DRS, might hinder translation of the results to the general population. Because of the preliminary character of this study, the sample size was predefined without estimating the minimum sample size. Such calculations require information about the extent of the measured trait variability in the population. To assess sample size in the methodologically correct way, it should be estimated on the basis of the previous scientific data. In our case, in the absence of other studies on peripheral activation of cellular components of the immune system in relation to the DRS, it correct and reliable calculations of minimum sample size were significantly impeded.

Activation of immune mechanisms in other oral diseases, namely periodontitis, has been observed previously, as elevated levels of C-reactive protein in periodontal patients have been reported [2]. This association was further confirmed by showing that C-reactive protein levels are lowered after periodontal treatment [8,39]. Since the DRS shares certain features with periodontal disease, such as the

presence of chronic local inflammation and involvement of the microorganisms [15], it can be hypothesised that there will be a similar effect on the systemic immune mechanisms. A recently published study has shown an association between DRS and vascular dysfunction [26], which was also observed for periodontitis [8,39]. This finding seems to support this theory, however common risk factors for oral and vascular diseases might confound interpretation of these results. The mechanisms of association between periodontitis and vascular diseases are not clearly defined, but are most likely dependent on the systemic inflammatory response, involving increased levels of IL-6, C-reactive protein, tumour necrosis factor-α and other cytokines, which accompanies periodontitis [35,50]. Cellular immunity is also activated in periodontitis, including monocyte subpopulation shifts, as well as T cell activation with overproduction of interferon gamma and IL-17 [5]. We therefore measured the impact of the treatment of oral fungal infection (DRS) on systemic features of T cells and monocytes activation in the short and medium term (2 months).

Contrary to our hypothesis, we did not observe any in the percentages of lymphocytes and T cells present in the peripheral blood of DRS patients in comparison to baseline values after treatment of DRS. The same observation was also true for CD4+ and CD8+ T cell subsets. The lack of differences in CD4+ T cell subset was the most surprising as this lymphocyte subset is thought to be of great importance in *Candida* immunity [53]. However, studies postulating pronounced role of CD4+ T cells in candidiasis have been performed mostly in immunocompromised individuals, while in our study population immune functions were not suppressed. It is therefore probable that the immune response may present different characteristics.

We studied naive (CD45RA+), memory (CD45RO+) and CD28null T cells subsets, because asymptomatic DRS is able to persist untreated for long periods of time and it is possible that such persistent infection may provoking continuous immune activation accelerating senescence of CD8+ T cells marked by loss of CD28 marker [52]. However, we observed changes in percentage of central memory (CD45RA-CCR7+) CD4+ T cells, which seem to decrease after treatment. These cells take part in immune response to the known pathogen and readily proliferate and differentiate to effector cells in response to antigenic stimulation [30,45], so after depleting of antigen source by defeating of chronic *Candida* infection, their amount decrease, reaching lower, stable levels.

CCR5 gene expression was shown to be enhanced in peripheral blood monocytes stimulated with *C. albicans in vitro* [20] and PBMC were shown to secrete RANTES in response to the presence of *C. albicans* [17]. We therefore studied the expression of the chemokine RANTES receptor - CCR5 on T cells. We observed similar pattern of CCR5 expression on the whole population of T cells and its particular subsets - CD4+ and CD8+. Lower expression was observed after 2 months after finishing the treatment, preceded by significant increase immediately after therapy. CCR5 is a marker of Th1 immune response and its ligand - RANTES leads immune cells to the site of inflammation. Resolution of DRS inflammation leads to a decreased amount of CCR5+ cells, and its initial increase might be related to the local effects of nystatin, including disruption of fungal cell integrity leading to enhanced exposure of its antigens and induction of proinflammatory IL-1β production [4,41], similarly to described later in this text intermediate monocyte subpopulation.

Consistently, we observed an increase in the percentage of CD69+ T cells and CD8+, but not CD4+, immediately after treatment, with a tendency to decrease 2 months after treatment which was statistically significant for the whole T cell population. Lower CD69 expression in response to *Candida*

stimulus between CD4+ and CD4- cells have been described before and reported percentages (1.5% and 10% for CD4+ and CD4- subsets, respectively) are consistent with our study [28]. CD69 is expressed on lymphocytes rapidly after activation and thus CD69 is known as marker of early lymphocyte activation. Therefore, it may seem surprising that the percentage of CD69 expressing cells increase after therapy, which lead to the resolving of inflammation. One possible explanation could be stimulating effects of nystatin on T cell activation as described for CCR5+ cells [4,41]. Another theory relies on the fact that CD69 may be implicated in memory T cell formation [27] and probably participate in the formation of immune memory after resolving of the infection.

DRS presence probably influences cytokine production by peripheral immune cells, as suggested by *in vitro* production of IL-2 by mononuclear blood cells of DRS patients in response to the *Candida albicans* [43] and high levels of IL-6 and tumour necrosis factors alpha observed in serum of DRS patients [40]. In contrast to these reports, we did not observe significant changes in production of intracellular proinflammatory cytokines, including IL-4, IL-17, tumour necrosis factors alpha or interferon gamma by T cell subsets after treatment of DRS. Our results are consistent with studies reporting no difference in cytokine concentration in saliva of DRS and healthy subjects [24]. Although differences in IL-17 production by CD4 T cells did not reach significance, they were close to it with p value of 0.061, pointing to a possible trend toward lowering IL-17 levels during subsequent study visits. Since the Th17 response, in which IL-17 is involved, is reported to be of particular importance in defence against fungal pathogens [10,34], it is possible that resolving the infection is followed by a decrease in IL-17 production. It is plausible that with larger study group, these differences would reach significance.

We also compared the amount of monocytes and their subpopulations, since these cells and their differentiated form - macrophages are elements of the first line of immunologic defence against an invading pathogen. Resolution of local inflammation in the oral cavity after treatment of DRS, was not associated with changes in percentage of monocytes. However, monocytes are very well known to play important role in inflammatory diseases such as rheumatoid arthritis or atherosclerosis [16,21]. It appears that especially CD16+ subsets are vital and their role in inflammatory conditions were described previously in end stage renal disease and in active rheumatoid arthritis [19,21]. Changes in monocyte subpopulations are visible even at a very early stage of disease [22], with increased amount of intermediate CD14highCD16+ and decreased amount of non-classical (CD14dimCD16+) monocytes. It is particularly interesting that we observed a reduction in the amount of classical CD14highCD16- and an increase in the amount of intermediate CD14highCD16+ monocyte subpopulations immediately after treatment and altered amount of these cells was observed to be sustained for at least two months. Classical monocyte subset are suggested as a potent inducer of Th17 immune response against *Candida albicans* [48]. This type of immune response is now considered vital in combating fungal infections [37]. After successful DRS therapy linked with reduction in numbers of *Candida* cells there was a reduction in CD14highCD16- monocytes indicating that induction of immune response was no longer present. The increase in intermediate monocyte subpopulation after therapy might be considered as surprising considering the proinflammatory and proatherogenic character of these cells [54], as well as an increase in CD14+CD16+ monocyte subset in periodontal disease [32]. The potential explanation of this observation includes two factors. Polyene macrolides antifungal drugs, such as nystatin, cooperate with host immune defence in clearance of the pathogen. They affect fungal cells directly by disrupting cell integrity [42] providing more ligands for host receptors involved in immune activation, such as pattern recognition receptors, thus transiently enhancing local inflammation. Although nystatin is considered as acting only topically and not being absorbed through skin and gastrointestinal tract, it is

also known to induce proinflammatory IL-1β production [4,41]. Mechanism of this activation involve Toll-like receptor 2 [41], which is also present on immune cells in oral mucosa [9,34]. It is possible, that these two processes temporarily enhance inflammation in treated area. Intermediate subset of monocytes are known to be increased in other inflammatory conditions [19,21], and this may also be the case in DRS.

To conclude, the main observation of our work is the lack of the evidence of a systemic cellular response to the treatment of oral *C. albicans* infection. This is in spite the fact that there is evidence of very complex immune mechanisms involved in defence against oral fungal infection including various cell types [33,46,51,53]. While this is in contrast to our hypothesis, we believe that this is a very important consideration for interpretation of some of systemic features of DRS. Simultaneously, our results might point to the possible, systemic inflammatory response to the topical application of polyene macrolide antifungal agent - nystatin. Although transient and non intense, such effect is mechanistically plausible and may comprise a clinically important finding. DRS patients are usually elderly and, as such, more susceptible to changes in immune function. Since IL-1β is a cytokine with proinflammatory characteristics, even low but unphysiologically induced production in susceptible population might influence the risk of inflammatory diseases, such as atherosclerosis [16,31] or rheumatoid arthritis [55]. Our previous study, where we described dysfunction of the vascular endothelium in DRS patients [26], may further point to the importance of this aspect, since endothelial dysfunction is known to precede the development of atherosclerosis [14] and its complications, such as hypertension.

Acknowledgments

We thank Dr Agnieszka Sagan and Dr Ryszard Nosalski from the Department of Internal and Agricultural Medicine, Jagiellonian University Medical College, Cracow, Poland for their valuable assistance in analysis of flow cytometry results and Dr Grzegorz Wilk from the same Department for his valuable clinical assistance. This study was supported by Public Funds for Science of Republic of Poland grant Nr 2997/B/P01/2009/36 and the Foundation for Polish Science Welcome Grant (FNP/Welcome/02) (TM, GO).

References

1. Abaci O, Haliki-Uztan A, Ozturk B, Toksavul S, Ulusoy M, Boyacioglu H. Determining Candida spp. incidence in denture wearers. Mycopathologia 2010;169:365–72.

2. Amar S, Gokce N, Morgan S, Loukideli M, Van Dyke TE, Vita JA. Periodontal disease is associated with brachial artery endothelial dysfunction and systemic inflammation. Arterioscler Thromb Vasc Biol 2003;23:1245–9.

3. Campos MS, Marchini L, Bernardes LAS, Paulino LC, Nobrega FG. Biofilm microbial communities of denture stomatitis. Oral Microbiol Immunol 2008;23:419–24.

4. Darisipudi MN, Allam R, Rupanagudi KV, Anders H-J. Polyene macrolide antifungal drugs trigger interleukin-1β secretion by activating the NLRP3 inflammasome. PloS One 2011;6:e19588.

5. Di Benedetto A, Gigante I, Colucci S, Grano M. Periodontal disease: linking the primary inflammation to bone loss. Clin Dev Immunol 2013;2013:503754.

6. Dorocka-Bobkowska B, Zozulinska-Ziolkiewicz D, Wierusz-Wysocka B, Hedzelek W, Szumala-Kakol A, Budtz-Jörgensen E. Candida-associated denture stomatitis in type 2 diabetes mellitus. Diabetes Res Clin Pract 2010;90:81–6.

7. Du Y, Heidemann C, Gößwald A, Schmich P, Scheidt-Nave C. Prevalence and comorbidity of diabetes mellitus among non-institutionalized older adults in Germany - results of the national telephone health interview survey "German Health Update (GEDA)" 2009. BMC Public Health 2013;13:166.

8. Elter JR, Hinderliter AL, Offenbacher S, Beck JD, Caughey M, Brodala N, et al. The effects of periodontal therapy on vascular endothelial function: a pilot trial. Am Heart J 2006;151:47.

9. Feller L, Khammissa R a. G, Chandran R, Altini M, Lemmer J. Oral candidosis in relation to oral immunity. J Oral Pathol Med 2014;43:563–9.

10. Gaffen SL, Herzberg MC, Taubman MA, Van Dyke TE. Recent advances in host defense mechanisms/therapies against oral infectious diseases and consequences for systemic disease. Adv Dent Res 2014;26:30–7.

11. Gasparoto TH, Sipert CR, de Oliveira CE, Porto VC, Santos CF, Campanelli AP, et al. Salivary immunity in elderly individuals presented with Candida-related denture stomatitis. Gerodontology 2012;29:e331–9.

12. Gendreau L, Loewy ZG. Epidemiology and etiology of denture stomatitis. J Prosthodont 2011;20:251–60.

13. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog 2010;6:e1000713.

14. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, et al. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. Circ Res 2000;86:E85–90.

15. Hajishengallis G. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. Trends Immunol 2014;35:3–11.

16. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685–95.

17. Huang C, Levitz SM. Stimulation of macrophage inflammatory protein-1alpha, macrophage inflammatory protein-1beta, and RANTES by Candida albicans and Cryptococcus neoformans in peripheral blood mononuclear cells from persons with and without human immunodeficiency virus infection. J Infect Dis 2000;181:791–4.

18. Huber CA, Schwenkglenks M, Rapold R, Reich O. Epidemiology and costs of diabetes mellitus in Switzerland: an analysis of health care claims data, 2006 and 2011. BMC Endocr Disord 2014;14:44.

19. Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, et al. CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum 2002;46:2578–86.

20. Kim HS, Choi EH, Khan J, Roilides E, Francesconi A, Kasai M, et al. Expression of genes encoding innate host defense molecules in normal human monocytes in response to Candida albicans. Infect Immun 2005;73:3714–24.

21. Kinne RW, Bräuer R, Stuhlmüller B, Palombo-Kinne E, Burmester G-R. Macrophages in rheumatoid arthritis. Arthritis Res 2000;2:189–202.

22. Klimek E, Mikołajczyk T, Sulicka J, Kwaśny-Krochin B, Korkosz M, Osmenda G, et al. Blood monocyte subsets and selected cardiovascular risk markers in rheumatoid arthritis of short duration in relation to disease activity. Biomed Res Int 2014;2014:736853.

23. Kossioni AE. The prevalence of denture stomatitis and its predisposing conditions in an older Greek population. Gerodontology 2011;28:85–90.

24. Leigh JE, Steele C, Wormley F, Fidel PL. Salivary cytokine profiles in the immunocompetent individual with Candida-associated denture stomatitis. Oral Microbiol Immunol 2002;17:311–4.

25. Lugo A, La Vecchia C, Boccia S, Murisic B, Gallus S. Patterns of smoking prevalence among the elderly in Europe. Int J Environ Res Public Health 2013;10:4418–31.

26. Maciąg J, Osmenda G, Nowakowski D, Wilk G, Maciąg A, Mikołajczyk T, et al. Denture-related stomatitis is associated with endothelial dysfunction. Biomed Res Int 2014;2014:474016.

27. Mackay LK, Braun A, Macleod BL, Collins N, Tebartz C, Bedoui S, et al. Cutting Edge: CD69 Interference with Sphingosine-1-Phosphate Receptor Function Regulates Peripheral T Cell Retention. J Immunol 2015;194:2059–63.

28. Maino VC, Suni MA, Ruitenberg JJ. Rapid flow cytometric method for measuring lymphocyte subset activation. Cytometry 1995;20:127–33.

29. Martori E, Ayuso-Montero R, Martinez-Gomis J, Viñas M, Peraire M. Risk factors for denture-related oral mucosal lesions in a geriatric population. J Prosthet Dent 2014;111:273–9.

30. Masopust D, Picker LJ. Hidden memories: Front line memory T cells and early pathogen interception. J Immunol 2012;188:5811–7.

31. McCarty S, Frishman W. Interleukin 1β: a proinflammatory target for preventing atherosclerotic heart disease. Cardiol Rev 2014;22:176–81.

32. Nagasawa T, Kobayashi H, Aramaki M, Kiji M, Oda S, Izumi Y. Expression of CD14, CD16 and CD45RA on monocytes from periodontitis patients. J Periodont Res 2004;39:72–8.

33. Naglik JR, Moyes D. Epithelial cell innate response to Candida albicans. Adv Dent Res 2011;23:50–5.

34. Naglik JR, Moyes DL, Wächtler B, Hube B. Candida albicans interactions with epithelial cells and mucosal immunity. Microbes Infect 2011;13:963–76.

35. Noh MK, Jung M, Kim SH, Lee SR, Park KH, Kim DH, et al. Assessment of IL-6, IL-8 and TNF-α levels in the gingival tissue of patients with periodontitis. Exp Ther Med 2013;6:847–51.

36. Oliveira MAM de, Carvalho LP, Gomes M de S, Bacellar O, Barros TF, Carvalho EM. Microbiological and immunological features of oral candidiasis. Microbiol Immunol 2007;51:713–9.

37. Pandiyan P, Conti HR, Zheng L, Peterson AC, Mathern DR, Hernandez-Santos N, et al. CD4+ CD25+ Foxp3+ regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17 cell infection model. Immunity 2011;34:422–34.

38. Perk J, De Backer G, Gohlke H, Graham I, Reiner Z, Verschuren WMM, et al. European Guidelines on cardiovascular disease prevention in clinical practice (version 2012): The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). Atherosclerosis 2012;223:1–68.

39. Piconi S, Trabattoni D, Luraghi C, Perilli E, Borelli M, Pacei M, et al. Treatment of periodontal disease results in improvements in endothelial dysfunction and reduction of the carotid intima-media thickness. FASEB J 2009;23:1196–204.

40. Pietruski JK, Pietruska MD, Jabłońska E, Sacha P, Zaremba M, Stokowska W. Interleukin 6, tumor necrosis factor alpha and their soluble receptors in the blood serum of patients with denture stomatitis and fungal infection. Arch Immunol Ther Exp (Warsz) 2000;48:101–5.

41. Razonable RR, Henault M, Watson HL, Paya CV. Nystatin induces secretion of interleukin (IL)-1beta, IL-8, and tumor necrosis factor alpha by a toll-like receptor-dependent mechanism. Antimicrob Agents Chemother 2005;49:3546–9.

42. Récamier KS, Hernández-Gómez A, González-Damián J, Ortega-Blake I. Effect of membrane structure on the action of polyenes: I. Nystatin action in cholesterol- and ergosterol-containing membranes. J Membr Biol 2010;237:31–40.

43. Rodriguez-Archilla A, Urquia M, Cutando A, Asencio R. Denture stomatitis: quantification of interleukin-2 production by mononuclear blood cells cultured with Candida albicans. J Prosthet Dent 1996;75:426–31.

44. Salerno C, Pascale M, Contaldo M, Esposito V, Busciolano M, Milillo L, et al. Candida-associated denture stomatitis. Med Oral Patol Oral Cir Bucal 2011;16:e139–43.

45. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745–63.

46. Schaller M, Boeld U, Oberbauer S, Hamm G, Hube B, Korting HC. Polymorphonuclear leukocytes (PMNs) induce protective Th1-type cytokine epithelial responses in an in vitro model of oral candidosis. Microbiology (Reading, Engl) 2004;150:2807–13.

47. Schmidt J, Jentsch H, Stingu C-S, Sack U. General immune status and oral microbiology in patients with different forms of periodontitis and healthy control subjects. PloS One 2014;9:e109187.

48. Smeekens SP, van de Veerdonk FL, Joosten LAB, Jacobs L, Jansen T, Williams DL, et al. The classical $CD14^{++}$ $CD16^{-}$ monocytes, but not the patrolling $CD14^{+}$ $CD16^{+}$ monocytes, promote Th17 responses to Candida albicans. Eur J Immunol 2011;41:2915–24.

49. Surdacki A, Sulicka J, Korkosz M, Mikolajczyk T, Telesinska-Jasiówka D, Klimek E, et al. Blood monocyte heterogeneity and markers of endothelial activation in ankylosing spondylitis. J Rheumatol 2014;41:481–9.

50. Tonetti MS, D'Aiuto F, Nibali L, Donald A, Storry C, Parkar M, et al. Treatment of periodontitis and endothelial function. N Engl J Med 2007;356:911–20.

51. Upadhyay J, Upadhyay RB, Agrawal P, Jaitley S, Shekhar R. Langerhans Cells and Their Role in Oral Mucosal Diseases. N Am J Med Sci 2013;5:505–14.

52. Vallejo AN, Weyand CM, Goronzy JJ. T-cell senescence: a culprit of immune abnormalities in chronic inflammation and persistent infection. Trends Mol Med 2004;10:119–24.

53. Villar CC, Dongari-Bagtzoglou A. Immune defence mechanisms and immunoenhancement strategies in oropharyngeal candidiasis. Expert Rev Mol Med 2008;10:e29.

54. Zawada AM, Rogacev KS, Schirmer SH, Sester M, Böhm M, Fliser D, et al. Monocyte heterogeneity in human cardiovascular disease. Immunobiology 2012;217:1273–84.

55. Zhao R, Zhou H, Su SB. A critical role for interleukin-1β in the progression of autoimmune diseases. Int Immunopharmacol 2013;17:658–69.

56. Zomorodian K, Haghighi NN, Rajaee N, Pakshir K, Tarazooie B, Vojdani M, et al. Assessment of Candida species colonization and denture-related stomatitis in complete denture wearers. Med Mycol 2011;49:208–11.

Table 1. Clinical characteristics of studied group. Data are presented as mean(SD) or n(%).

	DRS patients
Number of patients	15
Age [years]	62,5 (6,3)
BMI	29,1 (6,1)
Gender (M:F)	1 : 14
Current smoking	5 (33,3%)
Diabetes	5 (33,3%)
Coronary artery disease	6 (40%)
Controlled hypertension	6 (40%)
Untreated hypertension	8 (53,3%)
Newton's classification scale	
type I	0
type II	14
type III	1

BMI - body mass index; M - males; F - females

Figure 1. Maciąg et al

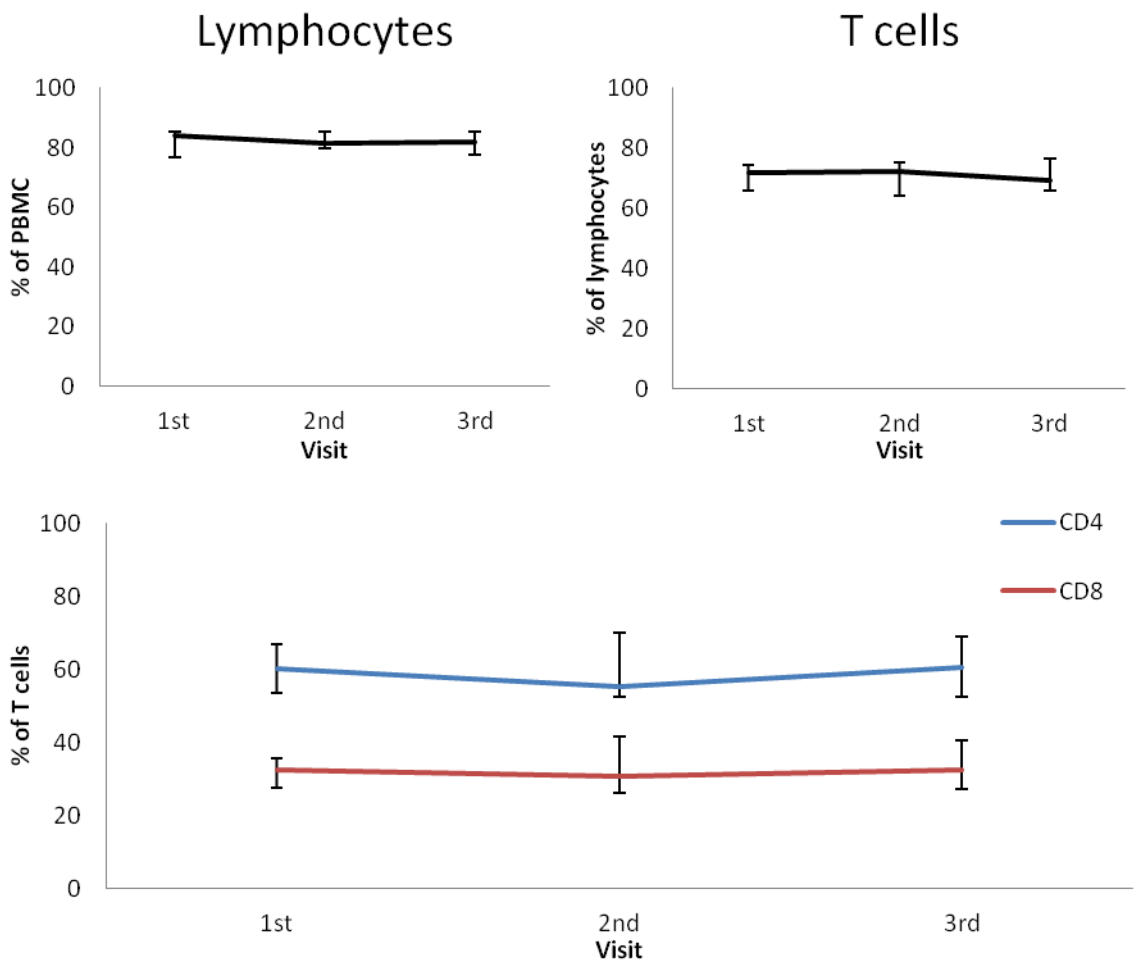

Figure 2. Maciąg et al

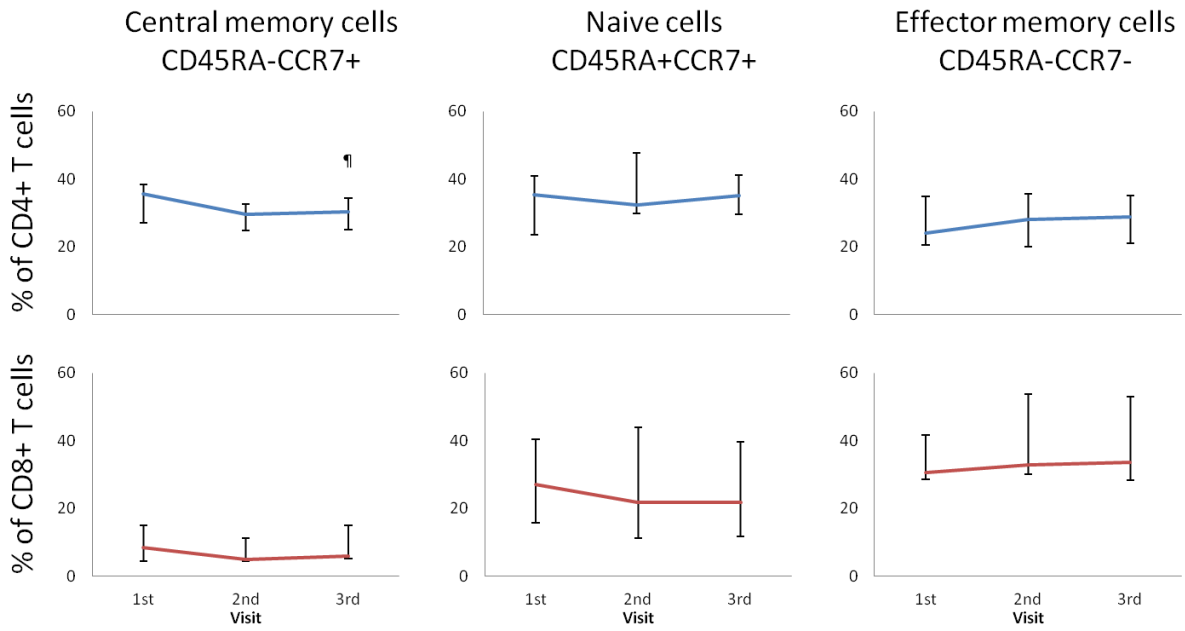

Figure 3. Maciąg et al

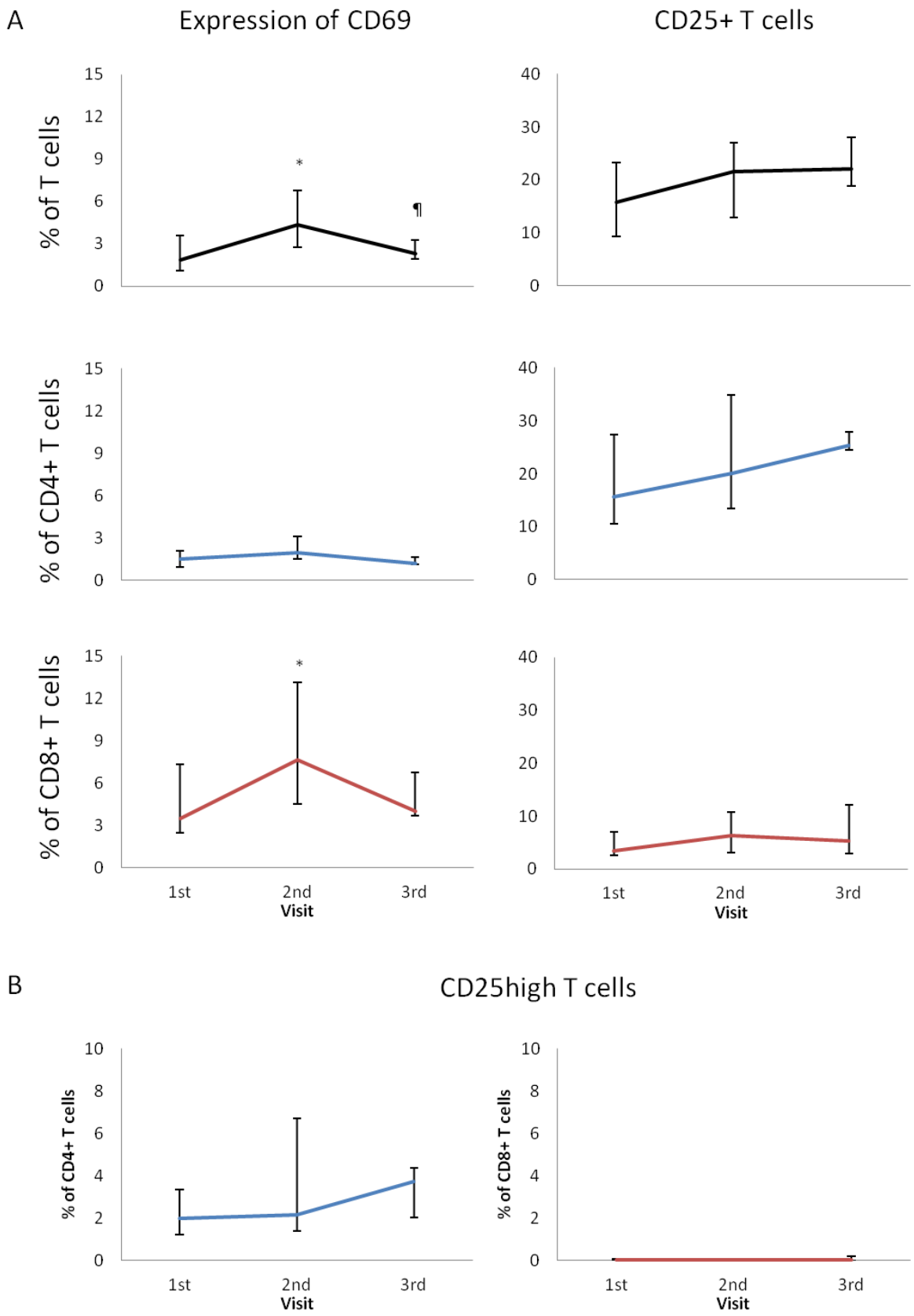

Figure 4. Maciąg et al

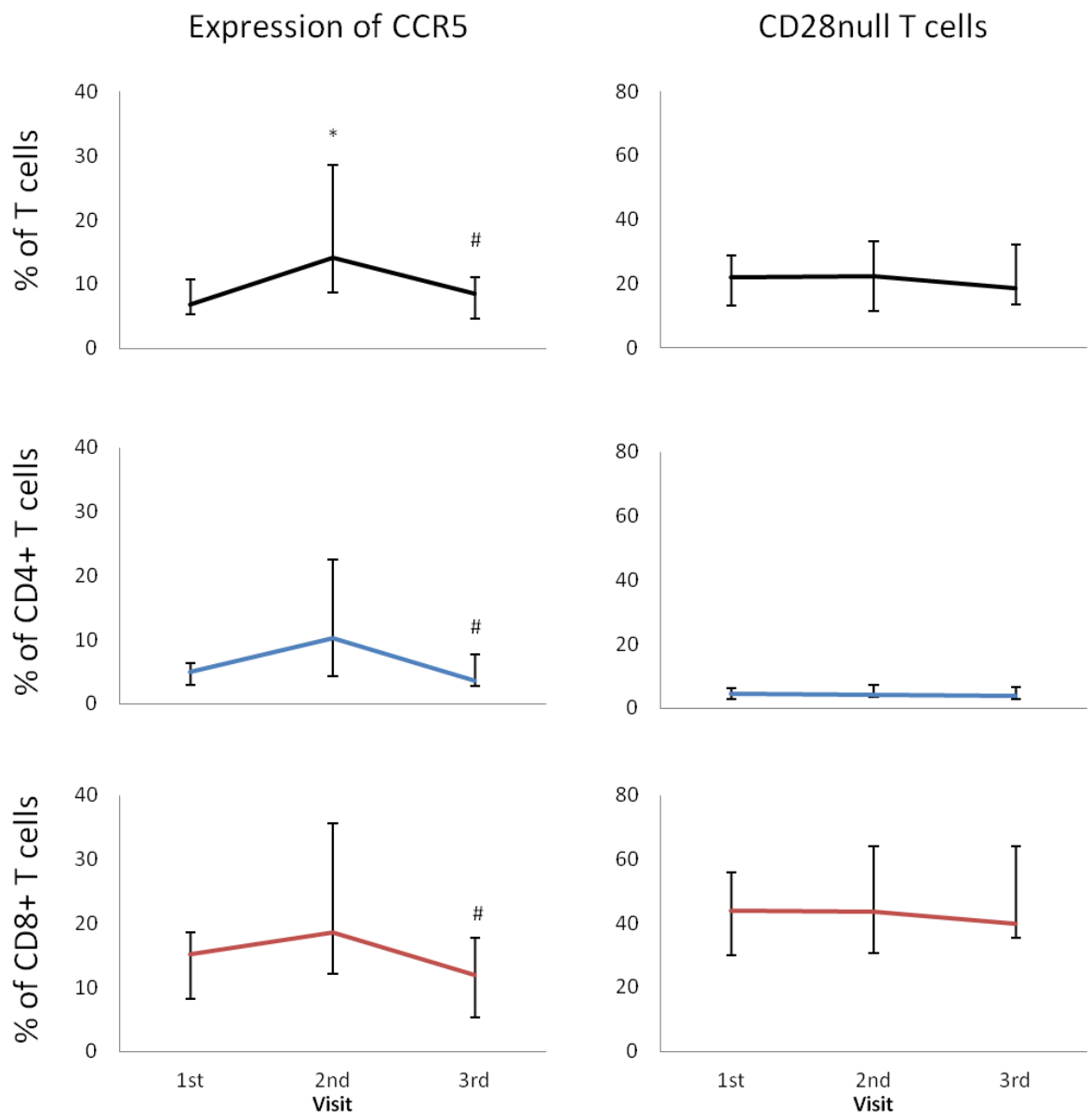

Figure 5. Maciąg et al

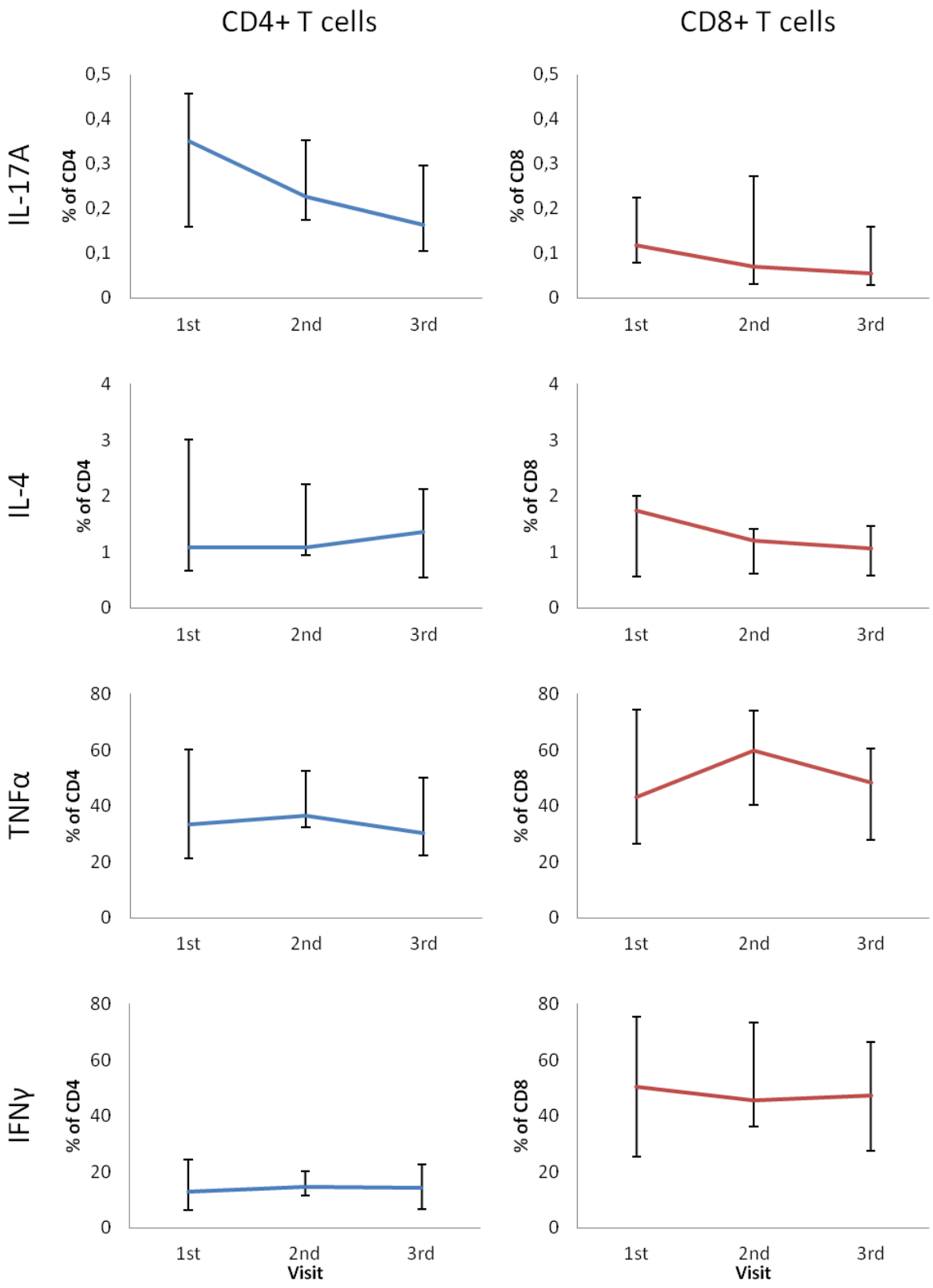

Figure 6. Maciąg et al

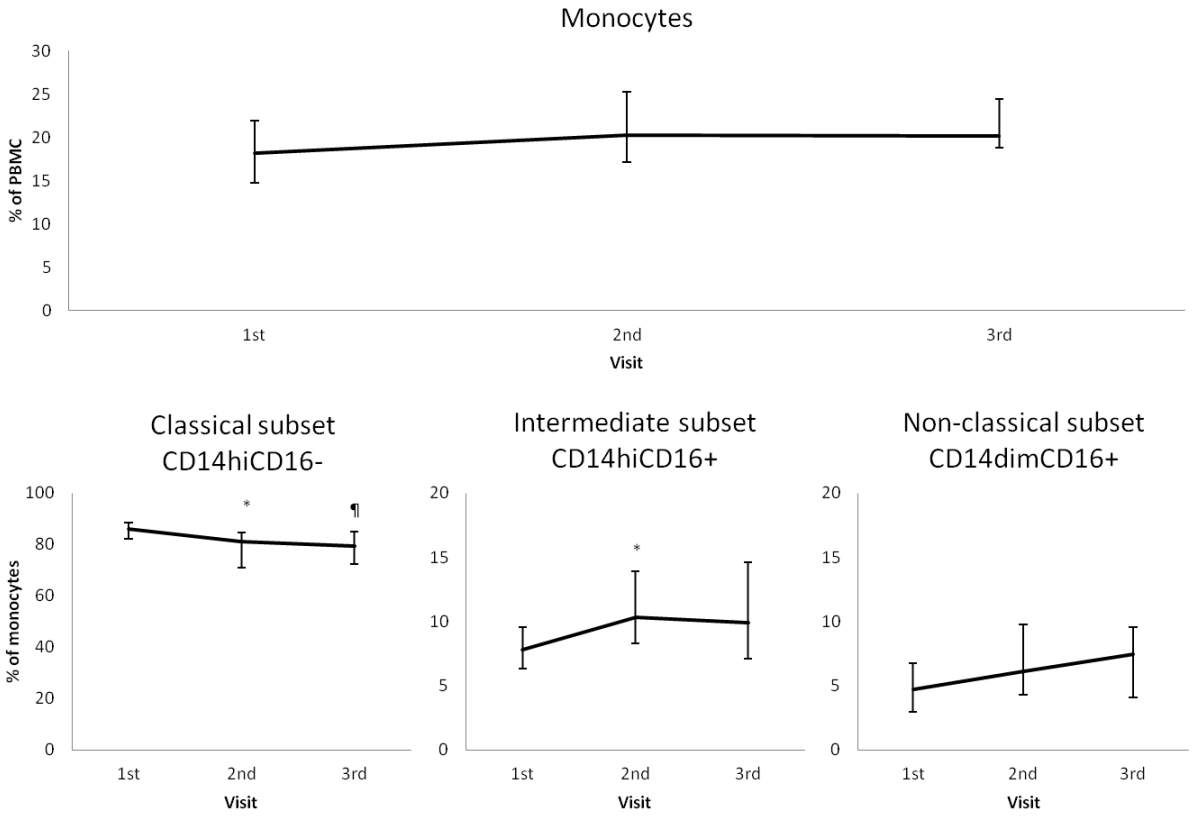

Figure legends:

Figure 1. Lymphocytes and their basic subpopulations in DRS patients treated with nystatin. Comparison of lymphocytes and T cells counts and basic T cell subpopulations: CD4+ and CD8+ in DRS patients before starting nystatin therapy (visit 1), immediately after finishing it (visit 2) and two months after therapy completion (visit 3). Lymphocyte subpopulation was measured in peripheral blood by flow cytometry. Results presented as median (Q1; Q2).

Figure 2. Subsets of the naive, effector and central memory CD4+ and CD8+ T cells present in peripheral blood of DRS patients treated with nystatin. Counts of the naive (CD45RA+CCR7+), effector (CD45RA-CCR7-) and central memory (CD45RA-CCR7+) T cells before starting nystatin therapy (visit 1), immediately after finishing it (visit 2) and two months after therapy completion (visit 3) were assessed by flow cytometry. Results presented as median (Q1; Q2). ¶ - $p < 0,05$ for comparison of 1st and 3rd visit.

Figure 3. A. Comparison of percentage of activated T cells of DRS patients treated with nystatin in CD4 and CD8 subset of lymphocytes. Expression of early activation marker (CD69) and late activation marker (CD25) were studied in peripheral blood by flow cytometry before starting nystatin therapy (visit 1), immediately after finishig it (visit 2) and two months after therapy completion (visit 3). Data are presented as % of cells expressing individual marker. Results presented as median (Q1; Q2). * - $p < 0,05$ for comparison of 1st and 2nd visit; ¶ - $p < 0,05$ for comparison of 1st and 3rd visit. **B. Presence of CD25high T cells in treated DRS patients.** Percentage of CD25high T cell subsets were assessed before starting nystatin therapy (visit 1), immediately after finishing it (visit 2) and two months after therapy completion (visit 3). Results presented as median (Q1; Q2).

Figure 4. Comparison of percentage of CCR5+ and of CD28null T cells subsets in DRS patients treated with nystatin. Chemokine RANTES receptor CCR5 in CD4 and CD8 subsets and prevalence of CD4+ CD28null and CD8+CD28null cells were studied in peripheral blood by flow cytometry before starting nystatin therapy (visit 1), immediately after finishing it (visit 2) and two months after therapy completion (visit 3). Data are presented as median (Q1;Q2) % of cells expressing individual marker. * - $p < 0,05$ for comparison of 1st and 2nd visit; # - $p < 0,05$ for comparison of 2nd and 3rd visit.

Figure 5. Results of intracellular staining of CD4+ and CD8+ T cell subsets in peripheral blood of DRS patients treated with nystatin. Percentage of cells producing IL-4, IL-17, TNFα or IFNγ was assessed by flow cytometry before starting nystatin therapy (visit 1), immediately after finishing it (visit 2) and two months after therapy completion (visit 3). Results presented as median (Q1; Q2); n = 10.

Figure 6. Comparison of monocytes and monocyte subsets in peripheral blood of DRS patients treated with nystatin. Counts of the presented subpopulations were assessed by flow cytometry before starting nystatin therapy (visit 1), immediately after finishing it (visit 2) and two months after therapy completion (visit 3). Results presented as median (Q1; Q2); n = 14. * - $p < 0,05$ for comparison of 1st and 2nd visit; ¶ - $p < 0,05$ for comparison of 1st and 3rd visit.